Interference of Transferrin Isoform Types with Carbohydrate-deficient Transferrin Quantification in the Identification of Alcohol Abuse

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Background: Isoforms of transferrin interfere with measurement of carbohydrate-deficient transferrin (CDT) as a marker of heavy alcohol consumption. We evaluated the rate of inaccurate CDT results by immunoassays.

Methods: We studied 2360 consecutive sera (1614 individuals) submitted for CDT assay without clinical information as well as samples from 1 patient with a congenital disorder of glycosylation (CDG Ia) and from 6 healthy carriers of CDG Ia. The CDTect, %CDT-TIA, and new %CDT immunoassays were compared with HPLC (%CDT-HPLC). Transferrin isoform pattern were evaluated by isoelectric focusing (IEF).

Results: Transferrin BC and CD heterozygotes were found at frequencies of ~0.7% and ~0.2%, respectively. Another transferrin C subtype, where di- and trisialotransferrin partly coeluted (tentatively identified as C2C3), was observed in ~0.6%. Compared with the %CDT-HPLC method, the immunoassays often produced low results for transferrin BC and high results for transferrin CD and “C2C3”. A very high trisialotransferrin value (frequency ~1%) often produced high CDT immunoassay results. In four of six healthy carriers of CDG Ia, a- and disialotransferrin were highly increased and the HPLC and IEF isoform patterns were indistinguishable from those in alcohol abuse.

Conclusions: Rare transferrin isoform types and abnormal amounts of trisialotransferrin (total frequency ~2–3%) may cause incorrect determination of CDT with immunoassays. The observed variants were readily identified by HPLC and IEF, which can be recommended for verification of CDT immunoassay results in doubtful cases. In healthy carriers of CDG Ia, CDT is high by all assays.

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The iron-transport glycoprotein, transferrin, consists of a single polypeptide chain with two binding sites for iron and two N-linked oligosaccharide units of complex structure. Transferrin can be separated into several isoforms, based on differences in the carbohydrate structure and mainly the number of negatively charged terminal sialic acid residues (1, 2). Human transferrin also shows genetic polymorphism, with transferrin C being the most common phenotype in all populations, whereas allelic B (lower pl) and D (higher pl) variants, with a different primary structure but a normal set of carbohydrate chains, occur at low frequencies (3). In common transferrin C phenotype serum, tetrasialotransferrin, which contains two biantennary carbohydrate chains with a total of four terminal sialic acid residues, is the predominant isoform and usually accounts for ~75% of total transferrin (4) (Fig. 1). Tri- and pentasialotransferrin typically make up ~5% and ~15%, respectively, whereas di- and hexasialotransferrin occur at <2% each, and the remaining isoforms (a-, mono-, hepta-, and octasialotransferrin) occur at <1%.

Carbohydrate-deficient transferrin (CDT),5 which refers to an abnormal microheterogeneity of serum transferrin (originally defined as the sum of a-, mono-, and/or disialotransferrin), has emerged as a useful biochemical marker for identifying chronic alcohol abuse and monitoring abstinence (5, 6). Individuals who have been drinking high amounts of alcohol (~50–80 g/day) for a period of at least 2 weeks often show increased concentrations of

5 Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; CDG, congenital disorder of glycosylation; IEF, isoelectric focusing; and WB, Western blot.
transferrin molecules that lack one (disialotransferrin) or both (asialotransferrin) carbohydrate chains (7–9). During abstention, serum CDT declines, with a half-life of 1.5–2 weeks (5, 10), and the time to reach a stable baseline could be 1 month or longer (11). The biological mechanism by which alcohol causes an increase in CDT has not yet been clearly identified, but most likely involves interference with the enzymes responsible for glycosyl transfer (12, 13).

The major benefit of CDT compared with other laboratory methods used in routine clinical medicine to indicate prolonged excessive alcohol consumption, such as the mean corpuscular volume of erythrocytes and the concentrations of γ-glutamyltransferase, aspartate aminotransferase, and alanine aminotransferase, is its higher specificity for alcohol exposure (5, 14, 15). However, CDT values outside the reference interval are occasionally found even without prior heavy drinking. Reported risks of false-positive CDT results include severe hepatic failure (primary biliary cirrhosis, chronic viral hepatitis, and hepatocellular carcinoma) (16–21), congenital disorders of glycosylation (CDG; formerly known as carbohydrate-deficient glycoprotein syndromes) (22, 23), genetic transferrin D variants (24, 25), pregnancy (26), estrogen use (27, 28), iron-deficiency anemia (29), low ferritin (30), high total transferrin (31, 32), combined pancreas and liver transplantation (33), and possibly, use of antiepileptic medications (34), although this was not observed in a previous study (14). On the other hand, genetic transferrin B variants (24) and an abnormally low total transferrin concentration (32) may cause false-negative CDT results.

Of major importance, but unfortunately less emphasized, is that the risk for obtaining erroneous test results is highly dependent on the method used for quantification of CDT. For example, a very high total transferrin concentration might cause false-positive results, but this occurs mainly when the CDT content is expressed as an absolute amount and not in relation to total transferrin (29, 32, 35, 36). Another current problem with CDT quantification is the lack of standardization. Many different analytical techniques and methods have been, and are still, in routine use, and the transferrin isoforms covered by different CDT tests also vary considerably (Fig. 2).

The present study was undertaken to evaluate analytical causes of falsely high and falsely low CDT results in identification of chronic alcohol abuse and the risk for incorrect determination of CDT during routine measurement when immunologic methods for quantification of CDT are used. The test results obtained with three commercial immunoassays (CDTect®, %CDT-TIA, and the new %CDT version; all from Axis–Shield ASA) were compared with the relative amount of CDT as determined by HPLC (%CDT-HPLC) (10), which was used as the reference method.

**Materials and Methods**

**SERUM SAMPLES**

The serum samples used were those sent to the Karolinska Laboratory in Stockholm for routine quantification of CDT by the %CDT-HPLC method. The samples originated mainly from patients living in the central part of Sweden. All measurements were performed without

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*Variants of the %CDT-TIA and new %CDT immunoassays are distributed by Bio-Rad and Roche.*
knowledge of the clinical diagnosis. Serum samples were also obtained from one patient with CDG Ia with two verified mutations in the CDG Ia [phosphomannomutase 2 (PMM2)] gene (37, 38). Six clinically healthy parents of patients with CDG Ia were also included; all of them had a single mutation in the PMM2 gene (37, 38). The sera were stored at 4 °C when analyzed within 1 day and at −20 °C for longer times.

CDT MEASUREMENTS
All immunoassays involved an initial iron saturation of the serum sample, because the variable degree of transferrin iron saturation otherwise affects the charge and thereby the chromatographic elution (39). The CDT isoforms were separated on disposable anion-exchange chromatography microcolumns, and the eluted CDT isoforms were quantified using anti-transferrin antibodies. All measurements were carried out according to the manufacturers’ instructions, using single determinations. The volume of some serum samples was not sufficient to be used in all CDT methods.

CDTect was the first commercial test for CDT quantification and was launched in 1992. It is a RIA and measures the sum of α-, mono-, and part of disialotransferrin as an absolute amount (in units/L, with 1 unit of CDT equivalent to ~1 mg of transferrin; Fig. 2). Because of a sex-based difference in the baseline values of total transferrin and the α- and monosialo isoforms (4, 24), different upper reference limits are applied for men and women (<20 and <26 units/L, respectively).

The %CDT-TIA test (Cobas Mira application) is a turbidimetric immunoassay that measures the sum of α-, mono-, di-, and a portion (~50%) of trisialotransferrin (in percentages) relative to the amount of total transferrin (Fig. 2). The total concentration of serum transferrin is measured separately using the same anti-transferrin antibody. An upper reference limit of 6% for both men and women was originally proposed by the manufacturer, but several studies have indicated that this cutoff may be lowered with retained high specificity (31, 32, 34, 35). An upper reference limit of <5.5% was used in this study.

The new Axis–Shield %CDT assay (microtiter applica-

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Fig. 3. Isoform patterns for different transferrin variants obtained by the %CDT-HPLC method. Chromatograms were obtained with the following samples: a serum sample of the predominant transferrin C variant (A; open arrow, disialotransferrin; for comparison, this chromatogram is also shown as the dashed line in panels B–F); transferrin C serum from a heavy drinker with typical increases in α- and disialotransferrin (B; filled arrow, high asialotransferrin; open arrow, high disialotransferrin); a transferrin BC heterozygote (C; open arrow, disialotransferrin C; hatched arrow, mixture of disialotransferrin B and trisialotransferrin C); a transferrin CD heterozygote (D; open arrow, disialotransferrin D; hatched arrow, mixture of disialotransferrin C and trisialotransferrin D); a transferrin C2C3 variant (E; open arrow, disialotransferrin; filled arrow, high trisialotransferrin); and a serum sample with very high trisialotransferrin (F; hatched arrow, monosialotransferrin; open arrow, disialotransferrin; filled arrow, high trisialotransferrin).
tion) is a turbidimetric immunoassay that measures primarily α-, mono-, and disialotransferrin (Fig. 2). An upper reference limit of <3% was used in this study (40).

The %CDT-HPLC method measures disialotransferrin and, when present, asialotransferrin as the amounts relative to total transferrin (percentages of peak areas of α- and disialotransferrin relative to all transferrin peaks; Fig. 2), using valley–valley integration of α-, di-, and trisialotransferrin and baseline integration of the higher isoforms (mainly tetra- and pentasialotransferrin) (10). Quantification relies on the selective absorbance of the iron-transferrin complex at 460 nm. The routine upper reference limit, <1.2% (95% confidence interval, 1.1–1.4; Bäck et al., manuscript in preparation), was also used in this study.

ISOELECTRIC FOCUSING
Isoelectric focusing (IEF) of transferrin isoforms was performed as described previously, using preanalytical removal of albumin by treatment with Blue Sepharose (10). The IEF transferrin isofrom pattern was also identified by Western blot (IEF/WB) (23).

Results
Three main genetic transferrin variants (B, C, and D) were identified by the %CDT-HPLC method in the clinical routine serum samples. The frequency of transferrin variants was determined among 1614 different individuals (2360 consecutive serum samples, but in several cases 2 or more samples originated from the same donor). Transferrin BC heterozygotes occurred at a frequency of ~0.7% and transferrin CD heterozygotes at a frequency of ~0.2%. Another previously described isoform subtype (41), where the trisialotransferrin peak was slightly shifted to the left in the HPLC chromatogram (i.e., cathodally) and partly coeluting with disialotransferrin (Fig. 3E), was also identified at a frequency of ~0.6%. Reanalysis and comparison with new samples from the same donors confirmed that this change was not caused by poor resolution on the HPLC column or aging of the serum samples. On the basis of the transferrin isofrom pattern obtained by IEF, this type was tentatively identified as transferrin C2C3 (Fig. 4).

The isofrom patterns obtained by HPLC and IEF for different transferrin isoform types are shown in Figs. 3 and 4, respectively. Because carriers of B alleles encode transferrin isoforms that elute after the corresponding transferrin C isoforms in the HPLC chromatograms, transferrin BC heterozygotes showed a mixture of transferrin B and C isoforms. Accordingly, for the BC heterozygotes identified in this study, disialotransferrin B eluted at approximately the same position as trisialotransferrin C, and trisialotransferrin B coeluted with tetrasialotransferrin C, and so forth (Fig. 3C). Carriers of D alleles, on the other hand, encode transferrin isoforms that elute ahead of the corresponding transferrin C isoforms. Thus, for the transferrin CD heterozygotes observed in this study, disialotransferrin D eluted in front of disialotransferrin C.

Fig. 4. IEF analysis of serum samples from various transferrin (Tf) B, C, and D homo- and heterozygotes. Some serum samples were derived from alcohol abusers, which explains the high concentrations of α- and disialotransferrin.

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Fig. 5. Correlations between the α-, di-, and trisialo isoforms of serum transferrin. (A), correlation between amounts of asialo- and disialotransferrin in 121 serum samples. In the samples with detectable asialotransferrin (C), a significant correlation between α- and disialotransferrin was observed (r = 0.795; P < 0.0001; n = 53). (B), lack of correlation between amounts of disialo- and trisialotransferrin in 273 serum samples (r = 0.022; P = 0.714).
and trisialotransferrin D coeluted with disialotransferrin C in the HPLC chromatograms, and so forth (Fig. 3D).

The HPLC peak representing asialotransferrin was observed only in cases of high disialotransferrin (~1.5% and higher; Fig. 5A). In these samples, a good correlation between a- and disialotransferrin was observed ($r = 0.795$; $P < 0.0001$; $n = 53$). The mean ($\pm$ SD) relative amount of trisialotransferrin was $3.9\% \pm 1.2\%$ (range, 1.0–10.2%; median, 3.9%; $n = 273$) as determined with the %CDT-HPLC method using valley–valley integration of the peaks. There was no significant difference in the amount of trisialotransferrin between female and male donors, and no correlation with disialotransferrin was found ($r = 0.022$; $P = 0.714$; Fig. 5B). A few serum samples showed very low (frequency $< 2\%$) or high (frequency $> 7\%$) trisialotransferrin concentrations, defined as $< 2\%$ and $> 7\%$ of total transferrin, respectively. The isoform peak representing monosialotransferrin was observed only in serum samples with very high trisialotransferrin content (Fig. 3F).

Compared with the results of the %CDT-HPLC reference method, transferrin BC heterozygotes often produced low results and transferrin CD variants high test results with the CDTect, %CDT-TIA, and new %CDT immunoassays (Fig. 6). All three immunoassays also yielded high CDT values with transferrin C2C3 serum. However, it was often impossible to quantify disialotransferrin in this transferrin isoform type with the HPLC method because of the partial coelution with trisialotransferrin (Fig. 3E). In serum samples with very high relative amounts of trisialotransferrin, all three immunoassay methods sometimes gave high CDT results that were not associated with increased amounts of di- and asialotransferrin. In this respect, the new %CDT assay apparently performed better than %CDT-TIA (Fig. 6).

Serum from one patient with CDG Ia showed a dis-
tinctly abnormal HPLC peak pattern (Fig. 7B) with particularly increased α- and disialotransferrin, as described previously on IEF/WB \((22, 23)\). The CDT concentration was very high with CDTect (332 units/L) and %CDT-TIA (>35%). Serum from four of six healthy parents of CDG Ia patients showed increased concentrations with %CDT-HPLC (2.7–4.9%) and %CDT-TIA (7.4–9.3%), and all had increased concentrations with CDTect (21–73 units/L) and abnormal transferrin isoform pattern on IEF/WB \((22)\). Their HPLC (Fig. 7C) and IEF/WB isoform (22) patterns were indistinguishable from those occurring after chronic heavy drinking.

Storage of serum samples (low and high %CDT serum; \(n = 6\)) for 3 days at room temperature \((22 °C)\) produced no change in the transferrin isoform pattern or in the %CDT values obtained by HPLC. Treatment of serum samples for short time with neuraminidase (type II-a from *Vibrio cholerae*; Sigma) produced a peak pattern different from that observed in alcohol-dependent subjects (Fig. 7D). Moreover, the retention time for the expected disialo isoform was not identical to that of native disialotransferrin.

**Discussion**

From a clinical point of view, the important question when using serum CDT to indicate chronic alcohol abuse is whether an increased concentration is correlated with chronic heavy drinking. Here the major benefit of CDT compared with traditional tests such as γ-glutamyltranspeptidase, aspartate aminotransferase, alanine aminotransferase, and mean corpuscular volume of erythrocytes is the higher specificity for alcohol \((14, 15)\). Nevertheless, a drawback is the lack of standardization for CDT quantification.\(^7\) Various analytical techniques as well as different definitions of CDT have been and still are in routine use, and this occasionally hampers direct comparison of data between studies \((39)\).

The immunologic assays for CDT are very convenient when large numbers of samples are to be analyzed on a routine basis. A disadvantage is that they measure a CDT fraction and, unlike methods based on HPLC and IEF, do not distinguish single transferrin isoforms. For that reason, it is difficult to know whether an increased numeric result obtained by the immunoassays is really related to alcohol abuse or may result from genetic variants of transferrin or other chromatographic interference, such as a high concentration of trisialotransferrin and the associated increased amount of the monosialo isoform.

Genetic transferrin variants or other transferrin aber-

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\(^7\) A meeting on CDT standardization was held in Berlin, Germany, in May 2000 (chaired by Dr. J.-O. Jeppsson). The meeting gathered clinical chemists and alcohol researchers from seven European countries, who agreed on the development of a sensitive reference method for CDT quantification based on the HPLC technique and baseline integration of the transferrin isoform peaks.
rations are quite common in most populations (3). The vast majority represent variants of transferrin C, and >20 subtypes have been identified by IEF or electrophoresis, with C1, C2, and C3 being the most common. The reported frequencies of transferrin C1, C2, and C3 alleles in the study area (central Sweden) are 0.77, 0.13, and 0.09, respectively (42), and similar frequencies have been found in southern Finland (0.74, 0.10, and 0.13) (43) and Germany (0.78, 0.13, and 0.07) (44). However, the transferrin C subtypes show only minor changes in charge and have been reported not to interfere with the quantification of CDT by immunoassays (24) or HPLC (10). For example, the %CDT-HPLC method used in this study did not separate homozygous transferrin C1 from C2 serum, although a slightly broadened disialotransferrin peak was observed (data not shown). Nonetheless, the present results indicate that one transferrin C subtype, tentatively identified as transferrin C2C3, which was found in <1% of the examined population, could interfere with the measurement of CDT by causing high test results with the immunoassays.

The present results confirm that transferrin B and D variants may give rise to inaccurate determination of serum CDT when immunoassays for CDT are used in identification of chronic alcohol abuse. In this context, transferrin BC and CD heterozygotes regularly gave low and high CDT results, respectively, albeit not always values below and beyond the reference limits. These results are in agreement with a few observations with other CDT immunoassays (24, 45). It should be pointed out that the transferrin B and D alleles are rare and occurred in <1% of the examined population. In a smaller group of 254 randomly selected individuals from the southernmost part of Sweden, the frequency of the transferrin BC variant was slightly higher (~2%; Bäck et al., manuscript in preparation). The allele frequencies correspond to the distribution reported previously in Caucasians (24, 46); the transferrin B and D variants were, for example, found with a heterozygous frequency of 1.53% in Germany (44). Transferrin D variants are, however, more common in certain Asian, Black, and South American populations (3).

Transferrin B, D, and C2C3 sera were readily identified from a unique peak pattern by the %CDT-HPLC method. Because of the two alleles at the transferrin locus, it was also possible to estimate the total amount of disialotransferrin for the observed transferrin BC and CD heterozygotes by HPLC, but it should be pointed out that this may not be feasible for all transferrin variants. Accordingly, the amount of disialotransferrin could be calculated using approximately twice the value of disialotransferrin C for transferrin BC heterozygotes and twice the value of disialotransferrin D for transferrin CD heterozygotes. The correction factor of ~2 was confirmed by comparing the integrated area of the two tetrasialotransferrin peaks with the total transferrin concentration in the sample (data not shown). However, for the transferrin C2C3 subtype, it often was impossible to determine the amount of disialotransferrin also by %CDT-HPLC because of the very poor separation between the di- and trisialotransferrin peaks.

The peak in the HPLC chromatograms representing asialotransferrin was observed only in cases of a high concentration of disialotransferrin (~1.5% and higher), which is in line with previous observations (47). Accordingly, because this is above the clinical cutoff limit of the %CDT-HPLC method (1.2%), the mere presence of a detectable asialotransferrin peak in the HPLC chromatogram represents a useful indicator of chronic excessive drinking. The monosialotransferrin peak was observed only in a few cases in connection with a very high relative amount of trisialotransferrin, but not in cases of the high disialotransferrin typically seen in chronic alcohol consumers. This indicates that monosialotransferrin is much less, or not at all, related to alcohol exposure (4). Nevertheless, monosialotransferrin will always be included in the CDT fraction measured by the immunoassays currently on the market because this isoform elutes between a- and disialotransferrin on the microcolumns used to separate the CDT fraction from the major transferrin isoforms. This also means that, although the new %CDT immunoassay does not measure trisialotransferrin and thus, as demonstrated in this study, is less affected by very high amounts of trisialotransferrin than the %CDT-TIA assay, there is still a risk of obtaining a falsely high CDT value in identification of alcohol abuse in these cases because of the associated increased amount of monosialotransferrin.

Apart from genetic transferrin D variants, established causes of false-positive CDT results in identification of alcohol abuse are primary biliary cirrhosis and CDG (5, 16, 23, 25). Primary biliary cirrhosis is an autoimmune disease that affects predominantly women. However, more recent data indicate that primary biliary cirrhosis causes high CDT results mainly when the results are expressed as an absolute amount but not as the amount relative to total transferrin (35), indicating that this may be attributable to an increased serum transferrin concentration in these patients. CDG are a group of rare recessively inherited diseases with severe neurologic and/or systemic manifestations from early childhood (48, 49). Diagnosis is based on clinical features and biochemical and molecular genetic analysis, and immunoassays for CDT and/or IEF/WB of transferrin are used as diagnostic screening methods (23, 48, 50). The CDG Ia subtype studied was also easily identified from an abnormal HPLC peak pattern of transferrin isoforms. Four of the six examined healthy carriers of CDG Ia showed increased CDT values with %CDT-TIA and %CDT-HPLC, whereas CDTect and IEF/WB yielded abnormal results for all six. It has previously been reported that ~25% of healthy CDG Ia carriers have increased CDT and IEF/WB patterns indistinguishable from those produced by alcohol abuse (22). Accordingly, based on the estimated carrier frequency of ~0.007 for CDG Ia in the Swedish popula-
tion (37), a high CDT value from this cause would occur in ~1 of every 500 individuals.

A high CDT result with the immunoassays could theoretically be attributable to sialic acid depletion of serum transferrin as a result of contamination with various microbes (bacteria or viruses) that produce neuraminidase (51, 52). However, the transferrin isoform pattern observed by the %CDT-HPLC method after serum was treated with neuraminidase was easily distinguishable from native serum samples. The reason for this difference is most likely that neuraminidase produces a successive removal of terminal sialic acid residues (i.e., a cathodal shift of higher sialylated to less sialylated isoforms) (1) and does not give the alcohol-induced selective increases of di- and asialotransferrin produced by a lack of entire carbohydrate chains (7–9). An atypical transferrin HPLC peak pattern leading to inaccurate CDT results was reported in serum samples stored at room temperature for a “long time” (53), but whether this was actually attributable to neuraminidase of microbial origin is unknown. The present results, although performed on a limited number of samples, indicate that CDT is rather stable in serum stored at room temperature (54).

In conclusion, various rare genetic variants and isoform types of transferrin as well as serum from healthy carriers of CDG Ia may cause incorrect determination of CDT as a marker of chronic heavy alcohol consumption. This risk was partly dependent on the assay used for CDT quantification. In unexpected or doubtful cases of a high CDT result with the immunoassays, it is recommended that the result be verified by HPLC or possibly IEF (55). This is especially important if the test results could lead to serious consequences for the individual, such as in workplace testing or reissuing of driving licenses (56).

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